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(54) Title: TREATMENT AND PREVENTION OF VASCULAR DISEASE

(57) Abstract

The present invention relates to the discovery that a nucleic acid molecule as set forth in SEQ ID NO:1 encodes a polypeptide (SEQ ID NO:2) that mediates the intracellular signal induced by the binding of TGFβ to specific cell surface receptors. In addition, it has been determined that the polypeptide of SEQ ID NO:2 also acts to increase the secretion of TGFβ. Moreover, the polypeptide (SEQ ID NO:4) encoded by the nucleic acid molecule as set forth in SEQ ID NO:3 was determined to modulate the activity of SEQ ID NO:2.

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TREATMENT AND PREVENTION OF VASCULAR DISEASE

BACKGROUND OF THE INVENTION

The vascular endothelium constitutes a major organ that functions as a regulator of blood coagulation, inflammation, and in the exchange of fluids and mediators between the intravascular compartment and parenchyma tissues. As such, the proper function of the endothelium is critical to overall homeostasis. A dysfunction of the endothelium resulting from an alteration in the expression of important surface molecules, can result in coagulation defects, local and systemic vascular inflammation, and enhancement in the progression and rupture of atherosclerotic plaque. These effects can further result in conditions including myocardial infarction, deep venous thrombosis, disseminated intravascular thrombosis, and stroke. Certain cell surface proteins are altered in response to a vascular injury or insult and can be used as markers of a dysfunctional endothelium. Two such factors are plasminogen activator inhibitor 1 (PAI-1) and thrombomodulin (TM)

PAI-1 plays a critical role in the fibrinolytic system by reducing the endogenous ability to remove fibrin by inhibiting plasminogen activators such as tissue type plasminogen activator (tPA). Studies have documented that elevations of PAI-1 are associated with increased risk of deep venous thrombosis. Further, elevations in PAI-1 are found in patients suffering from myocardial infarction and septicemia. Because impaired fibrinolytic capacity is associated with increased cardiovascular risk, lowering PAI-1 should result in lowered risk.

While PAI-1 can be produced in a variety of tissues, substantial levels are secreted by the vascular endothelial cell. Since PAI-1 can be increased in

endothelial cells in response to certain stimuli, including cytokines, it contributes to dysfunction of the endothelium and the attendant problems.

TM plays a critical role in maintaining vessel anticoagulant activity as a cofactor for thrombin-catalyzed activation of protein C, the major endogenous antithrombotic factor. Like PAI-1, the surface anticoagulant responses can be impaired in states of endothelial dysfunction. In fact, TM levels become suppressed in cytokine activated endothelium.

Members of the transforming growth factor beta (TGFβ) superfamily are involved in many physiological activities including development, tissue repair, hormone regulation, bone formation, and cell growth and differentiation. Recent work has identified a family of related gene products called "Smads" that function downstream of the receptors of the TGFβ family. Upon activation, several of the Smads translocate directly to the nucleus where they activate transcription. The Smads have been implicated in the regulation of cell growth and proliferation, development and differentiation of cell types, and in endothelial cell response to shear stress. Smad1 is a bone morphogenetic protein (BMP) signal transducer, Smad2 and Smad3 are TGFβ and activin signal transducers, and Smad4 (also known as DPC4) is a tumor suppressor that functions as a mediator of TGFβ signaling.

Smad6 (SEQ ID NO:2) and Smad7 (SEQ ID NO:4) were initially discovered in endothelial cells to be implicated in cardiovascular disease (US Pat. No. 5,834,248 and WO 96/24604, each entirely incorporated by reference herein). Smad7 directly interferes with TGFβ mediated activation of Smad2 by preventing phosphorylation, interaction with Smad4, and nuclear accumulation.

Accordingly, there is a need to provide molecules, such as polypeptides, nucleic acids, and/or organic compounds that modulate expression, or at least one activity of, proteins such as Smad6 and Smad7, which molecules can be used to treat or affect cells, tissues, animals (e.g., humans) for various diseases or pathologies affected by such proteins.

SUMMARY OF THE INVENTION

Since the local control of PAI-1 and TM at the endothelial cell/plasma interface can play a major role in many pathological processes, use of Smad6 and Smad7 polypeptides or agents that modulate their expression and/or activity in the endothelium can be used to treat conditions such as, but not limited to, sepsis, injuries involving major tissue damage and trauma, systemic inflammatory response syndrome, sepsis syndrome, septic shock and multiple organ dysfunction syndrome (including DIC), as well as myocardial infarction, deep venous thrombosis, disseminated intravascular thrombosis, atherosclerotic plaque rupture, and associated sequela. Likewise, Smad6 and Smad7 polypeptides or other molecules that modulate their expression and/or activity can be used to prevent stroke. Further, because of the critical role of fibrin in tumor cell biology, Smad6 and Smad7 polypeptides or molecules that modulate their expression and/or activity can be used as anti-metastatic agents.

Moreover, the Smad6 polypeptide has been determined to increase expression of TGFβ. Smad6-induced TGFβ expression can be inhibited by inhibitors of protein kinase C (PKC).

The current invention provides methods for treating or preventing at least one aspect of at least one vascular disease; the method comprising administering to a patient in

need of such treatment, a compound that modulates at least one TGFβ regulated activity. Preferred TGFβ regulatable activities include but are not limited to an induction of plasminogen activator inhibitor-1 expression, an increase in plasminogen activator inhibitor-1 secretion, a suppression of thrombomodulin activity, an increase in TGFβ secretion, and a decrease in TGFβ secretion.

Further, the current invention provides in one aspect a method for treating or preventing a disease in which TGFβ is responsible for inducing cellular effects that lead to at least one disease state; the method comprising administering to a patient in need of such treatment, a compound that modulates expression of, or the activity of the protein product encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:1 (encoding at least one Smad6 protein), SEQ ID NO:3 (encoding at least one Smad7 protein), and a nucleic acid molecule that is complementary to SEQ ID NO:1 or 3.

The current invention also provides methods for identifying compounds that modulate anticoagulant and fibrinolytic functions in the vasculature endothelium; the method comprising contacting at least one cell or an organism with a compound which modulates expression or activity of PAI-I and TM.

As part of the current invention, there is also provided methods for identifying compounds, such as polypeptides or organic molecules, that modulate the expression or activity of Smad6 or Smad7 (e.g., proteins or polypeptides encoded by SEQ ID NO:1 and 2, respectively), which in turn modulate expression or activity of anticoagulant and fibrinolytic functions, namely PAI-I and TM, in the vasculature endothelium.

The present invention also provides a method for modulating TGFβ regulatable activities comprising administering to a patient in need of such treatment, a

protein product encoded by a nucleic acid molecule having at least 70, 75, 80, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity to a member selected from the group consisting of at least 30 nucleotides of at least one of SEQ ID NO:1, SEQ ID NO:3, and a nucleic acid molecule that is complementary to SEQ ID NO:1 or SEQ ID NO:3. Preferred protein products for use in the current invention include SEQ ID NO:2 and SEQ ID NO:4. A nucleic acid molecule having at least 70% identity to the specified sequences are preferred for use in the present invention; identity of at least 95% is especially preferred. Preferred TGFβ regulatable activities include but are not limited to an induction of plasminogen activator inhibitor-1 expression, an increase in plasminogen activator inhibitor-1 secretion, a suppression of thrombomodulin activity, an increase in TGFβ secretion, and a decrease in TGFβ secretion.

The present invention also provides a method for modulating TGFβ regulatable activities comprising administering to a cell or cells, a protein product encoded by a nucleic acid molecule having at least 70, 75, 80, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity to a member selected from the group consisting of at least 30 nucleotides of at least one of SEQ ID NO:1, SEQ ID NO:3, and a nucleic acid molecule that is complementary to SEQ ID NO:1 or SEQ ID NO:3. Preferred protein products for use in the current invention include SEQ ID NO:2 and SEQ ID NO:4. A nucleic acid molecule having at least 70% identity to the specified sequences are preferred for use in the present invention; identity of at least 95% is especially preferred. Preferred TGFβ regulatable activities include but are not limited to an induction of plasminogen activator inhibitor-1 expression, an increase in plasminogen activator inhibitor-1 secretion,

a suppression of thrombomodulin activity, an increase in TGFβ secretion, and a decrease in TGFβ secretion.

In another embodiment the present invention relates to a pharmaceutical formulation comprising as an active ingredient a Smad6 or Smad7 polypeptide, associated with one or more pharmaceutically acceptable carriers, excipients, or diluents thereof. Preferred protein products for use in the current invention include SEQ ID NO:2 and SEQ ID NO:4.

The current invention also provides a method for modulating TGFβ regulatable activities comprising administering to at least one cell, an organism, or a patient in need of such treatment, an antisense nucleic acid molecule having a nucleotide sequence complementary to a contiguous sequence of mRNA transcribed from a gene selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3, wherein said antisense nucleic acid molecule hybridizes to said contiguous sequence such that translation of said mRNA is inhibited. It is preferred if the contiguous sequence includes at least fifteen nucleotides. Preferred nucleic acid molecules for use in the current invention include SEQ ID NO:5 and SEQ ID NO:7. Preferred TGFβ regulatable activities include but are not limited to an induction of plasminogen activator inhibitor-1 expression, an increase in plasminogen activator inhibitor-1 secretion, a suppression of thrombomodulin activity, an increase in TGFβ secretion, and a decrease in TGFβ secretion.

The invention also provides methods for the identification of compounds that modulate a TGFβ regulatable activity comprising administering to at least one cell or an organism a compound that modulates expression of, or the activity of the protein product of, a nucleic acid molecule selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and fragments thereof. Preferred TGFβ regulatable activities include but are not limited to an induction of

plasminogen activator inhibitor-1 expression, an increase in plasminogen activator inhibitor-1 secretion, a suppression of thrombomodulin activity, an increase in TGFβ secretion, and a decrease in TGFβ secretion.

The present invention also provides methods for the identification of compounds that modulate a TGFβ regulatable activity comprising administering to at least one cell, or an organism a compound that results in an induction of plasminogen activator inhibitor-1 expression, an increase in plasminogen activator inhibitor-1 secretion, a suppression of thrombomodulin activity, an increase in TGFβ secretion, or a decrease in TGFβ secretion.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

The terms "complementary" or "complementarity" as used herein refer to the capacity of purine and pyrimidine nucleotides to associate through hydrogen bonding to form double stranded nucleic acid molecules. The following base pairs are related by complementarity: guanine and cytosine; adenine and thymine; and adenine and uracil. As used herein, "complementary" means that the aforementioned relationship applies to substantially all base pairs comprising two single-stranded nucleic acid molecules over the entire length of said molecules. "Partially complementary" refers to the aforementioned relationship in which one of two single-stranded nucleic acid molecules is shorter in length than the other such that a portion of one of the molecules remains single-stranded.

The term "conservative substitution" or "conservative amino acid substitution" refers to a replacement of one or more amino acid residue(s) in a parent polypeptide as stipulated by Table 1.

The term "fragment thereof" refers to a fragment, piece, or sub-region of a nucleic acid or polypeptide molecule whose sequence is disclosed herein, such that the fragment comprises 5 or more amino acids, or 10 or more nucleotides that are contiguous in the parent polypeptide or nucleic acid molecule. "Fragment thereof" may or may not retain biological activity. A fragment of a polypeptide disclosed herein could be used as an antigen to raise a specific antibody against the parent polypeptide molecule. With reference to a nucleic acid molecule, "fragment thereof" refers to 10 or more contiguous nucleotides, derived from a parent nucleic acid. The term also encompasses the complementary sequence. For example if the fragment entails the sequence 5'-AGCTAG-3', then "fragment thereof" would also include the complementary sequence, 3'-TCGATC-5'.

The term "functional fragment" or "functionally equivalent fragment", as used herein, refers to a region, or fragment of a full length polypeptide, or sequence of amino acids that, for example, comprises an active site, or any other motif, relating to biological function. Functional fragments are capable of providing a substantially similar biological activity as a polypeptide disclosed herein, *in vivo* or *in vitro*, *viz.* the capacity to modulate cell proliferation. Functional fragments may be produced by cloning technology, or as the natural products of alternative splicing mechanisms.

The term "functionally related" is used herein to describe polypeptides that are related to the Smad6 or Smad7 polypeptides used in the present invention, said functionally related polypeptides constituting modifications of said Smad6 or Smad7 polypeptides, in which conservative amino acid changes are present as natural polymorphic variants of the polypeptides disclosed herein. Conservative amino acid substitutions and modifications may also be

engineered using recombinant DNA techniques. Functionally related polypeptides retain the biological activity of Smad6 or Smad7, such as the ability to inhibit cell proliferation, and/or tumor growth in vivo or in vitro.

The symbol "N" in a nucleic acid sequence refers to adenine ("A"), guanine ("G"), cytosine ("C"), thymine (T"), or uracil ("U"); "Z" designates an unknown amino acid residue.

The term "plasmid" refers to an extrachromosomal genetic element. The plasmids disclosed herein are commercially available, publicly available on an unrestricted basis, or can be constructed from readily available plasmids in accordance with published procedures.

A "primer" is a nucleic acid fragment which functions as an initiating substrate for enzymatic or synthetic elongation of, for example, a nucleic acid molecule.

The term "promoter" refers to a nucleic acid sequence that directs transcription, for example, of DNA to RNA. An inducible promoter is one that is regulatable by environmental signals, such as carbon source, heat, or metal ions, for example. A constitutive promoter generally operates at a constant level and is not regulatable.

The terms "protein" and "polypeptide" are used interchangeably herein and are intended to mean a biopolymer comprising a plurality of amino acid residues covalently bound in peptide linkage.

The term "recombinant DNA cloning vector" as used herein refers to any autonomously replicating agent, including, but not limited to, plasmids and phages, comprising a DNA molecule to which one or more additional DNA segments can or have been incorporated.

The term "recombinant DNA expression vector" or "expression vector" as used herein refers to any recombinant DNA cloning vector, for example a plasmid or phage, in which

a promoter and other regulatory elements are present thereby enabling transcription of an inserted DNA, which may encode a protein.

The term "substantially pure," used in reference to a polypeptide, means substantial separation from other cellular and non-cellular molecules, including other protein molecules. A substantially pure preparation would be about at least 85% pure; preferably about at least 95% pure. A "substantially pure" protein can be prepared by a variety of techniques, well known to the skilled artisan, including, for example, the IMAC protein purification method.

The term "vector" as used herein refers to a nucleic acid compound used for introducing exogenous or endogenous DNA into host cells. A vector comprises a nucleotide sequence which may encode one or more protein molecules. Plasmids, cosmids, viruses, and bacteriophages, in the natural state or which have undergone recombinant engineering, are examples of commonly used vectors.

The various restriction enzymes disclosed and described herein are commercially available and the manner of use of said enzymes including reaction conditions, cofactors, and other requirements for activity are well known to one of ordinary skill in the art. Reaction conditions for particular enzymes were carried out according to the manufacturer's recommendation.

In accordance with an aspect of the present invention, it has been discovered that a nucleic acid molecule as set forth in SEQ ID NO:1 encodes a polypeptide (SEQ ID NO:2) that mediates the intracellular signal induced by the binding of TGFβ to specific cell surface receptors.

The current invention arises from the discovery that Smad6 and Smad7 mediate the expression of anticoagulant and fibrinolytic functions in the vascular endothelium, as well as modulation of TGFβ secretion. Specifically, the over-expression of Smad6 increases the secretion of PAI-1

and suppresses the expression of TM (measured by the ability of cells to support thrombin-mediated activation of protein C), similar to the direct affect of TGFβ itself. Conversely, the inhibition of Smad6 by antisense decreases TGFβ-induced PAI-1 levels/activity and increases TGFβ-induced suppression of TM. Further, the effects of Smad6 on these coagulation markers can be antagonized by Smad7. These results with antisense and over-expression demonstrate that Smad6 can mimic a TGFβ response in the absence of added TGFβ, and is required for transducing the signal when TGFβ is added. In addition, Smad6 acts as a feedback amplifier of the pathway by up-regulating the secretion of TGFβ1 and TGFβ3. Protein kinase C and inhibitors thereof also modulate the activity of Smad6.

Other effects of inhibiting Smad6 expression and activity include blocking suppression of thrombomodulin, which in turn leads to an increase in activated protein C.

It has also been demonstrated by immunolocalization and *in situ* hybridization studies of the vasculature that Smad6 is predominately expressed in atherosclerotic lesions, but not in the normal endothelium, whereas Smad7 is predominately expressed in the normal endothelium but not in atherosclerotic lesions. Strikingly, Smad6 is also highly over-expressed in diseased heart, where TGFβ is known to play a critical role in congestive heart failure.

Smad7 inhibits type I receptor kinase signaling by acting as intracellular antagonist of the type I receptor domain. Smad7 interacts directly with the activated type I receptor, thereby blocking Smad2 phosphorylation, Smad2 association with Smad4, and nuclear accumulation of the complex.

Therefore, agents that suppress the level and/or function of Smad6, or increase the level and/or function of Smad7 would be useful agents to increase anticoagulant or

profibrinolytic functions in the vasculature for the prevention and treatment of myocardial infarction and peripheral vascular disease, as well as the prevention of vascular dysfunction and thromboembolytic contributions to stroke. Further, such agents would be useful in the prevention and treatment of cardiac hypertrophy and functional failure. Compounds that modulate expression of, or the activity of the protein product of, a nucleic acid molecule molecule selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and fragments or compounds having at least 90% identity to SEQ ID NO:2, SEQ ID NO:4, or fragments thereof can be used for the manufacture of a medicament for the treatment and/or prevention of diseases, including but not limited to, myocardial infarction, congestive heart failure, dilated cardiomyopathy, deep venous thrombosis, disseminated intravascular thrombosis, stroke, sepsis, injuries involving major tissue damage and trauma, systemic inflammatory response syndrome, sepsis syndrome, septic shock, multiple organ dysfunction syndrome (including DIC), atherosclerotic plaque rupture, and associated sequela arising therefrom.

Because of the critical importance of PAI-1 and TM in venous and microvascular functions, such agents would be particularly useful in the treatment of hypercoagulable states and disseminated intravascular coagulation associated with systemic inflammatory responses, sepsis, burns, obstetrical complications, and trauma.

For therapeutic use in preventing or treating vasculature disease an effective amount of Smad6 or Smad7 polypeptide is administered to an organism in need thereof in a dose between about 0.001 and 10,000 μg/kg body weight. In practicing the methods contemplated, Smad6 or Smad7 can be administered in a single daily dose or in multiple doses per day. The amount per administration will be determined by the physician and depend on such factors as the nature and

severity of the disease, and the age and general health of the patient.

The present invention also provides a pharmaceutical composition comprising as the active agent a Smad6 or Smad7 polypeptide or fragment thereof, or a pharmaceutically acceptable non-toxic salt thereof, and a pharmaceutically acceptable solid or liquid carrier. For example, compounds comprising Smad6 or Smad7 can be admixed with conventional pharmaceutical carriers and excipients, and used in the form of tablets, capsules, elixirs, suspensions, syrups, wafers, and the like. The compositions comprising Smad6 or Smad7 will contain from about 0.1% to 90% by weight of the active compound, and more generally from about 10% to 30%. The compositions may contain common carriers and excipients such as corn starch or gelatin, lactose, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride, and alginic acid. The compounds can be formulated for oral or parenteral administration.

For intravenous (IV) use, the Smad6 or Smad7 polypeptide is administered in commonly used intravenous fluid(s) and administered by infusion. Such fluids, for example, physiological saline, Ringer's solution or 5% dextrose solution can be used.

For intramuscular preparations, a sterile formulation, preferably a suitable soluble salt form of the Smad6 or Smad7 polypeptide, for example SEQ ID NO: 2 or SEQ ID NO: 4, such as the hydrochloride salt, can be dissolved and administered in a pharmaceutical diluent such as pyrogen-free water (distilled), physiological saline or 5% glucose solution. A suitable insoluble form of the compound may be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, e.g. an ester of a long chain fatty acid such as ethyl oleate.

Skilled artisans will recognize that the proteins used in the present invention can be synthesized by a number of different methods, such as chemical methods well known in the art, including solid phase protein synthesis or recombinant methods. Both methods are described in U.S. Patent 4,617,189, entirely incorporated herein by reference.

The principles of solid phase chemical synthesis of polyproteins are well known in the art and may be found in general texts in the area. *See, e.g.*, H. Dugas and C. Penney, Bioorganic Chemistry (1981) Springer-Verlag, New York, 54-92. For example, proteins may be synthesized by solid-phase methodology utilizing an Applied Biosystems 430A protein synthesizer (Applied Biosystems, Foster City, CA) and synthesis cycles supplied by Applied Biosystems.

The proteins used in the present invention can also be produced by recombinant DNA methods using a cloned or other Smad6 or Smad7 nucleic acid template. Recombinant methods are preferred if a high yield is desired. Expression of a Smad6 or Smad7 gene can be carried out in a variety of suitable host cells, well known to those skilled in the art. For example, the Smad6 or Smad7 gene or fragments thereof (e.g. SEQ ID NOS: 1 or 3) can be introduced into host cells by any suitable means well known to those skilled in the art. While chromosomal integration of the cloned genes is possible, it is preferred that the gene or fragment thereof be cloned into a suitable extra-chromosomally maintained expression vector so that the coding region of the Smad6 or Smad7 gene is operably-linked to a constitutive or inducible promoter.

The basic steps in the recombinant production of a Smad6 or Smad7 polypeptide are:

a) constructing a natural, synthetic or semi-synthetic DNA encoding a Smad6 or Smad7 polypeptide;

b) integrating said DNA into an expression vector in a manner suitable for expressing the Smad6 or Smad7 polypeptide, either alone or as a fusion protein;

c) transforming or otherwise introducing said vector into an appropriate eucaryotic or prokaryotic host cell forming a recombinant host cell,

d) culturing said recombinant host cell in a manner to express the Smad6 or Smad7 polypeptide; and

e) recovering and substantially purifying the Smad6 or Smad7 polypeptide by any suitable means, well known to those skilled in the art.

Procaryotes may be employed in the production of recombinant Smad6 or Smad7 polypeptide. For example, the *Escherichia coli* K12 strain 294 (ATCC No. 31846) is particularly useful for the prokaryotic expression of foreign proteins. Other strains of *E. coli,* bacilli such as *Bacillus subtilis,* enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcescens,* various *Pseudomonas* species and other bacteria, such as *Streptomyces,* may also be employed as host cells in the cloning and expression of the recombinant proteins of this invention.

Promoter sequences suitable for driving the expression of genes in procaryotes include β-lactamase (*e.g.* vector pGX2907 (ATCC 39344) which contains a replicon and β-lactamase gene), lactose systems (Chang et al., *Nature* (London), 275:615 (1978); Goeddel et al., *Nature* (London), 281:544 (1979)), alkaline phosphatase, and the tryptophan (trp) promoter system (vector pATH1 (ATCC 37695) which is designed to facilitate expression of an open reading frame as a trpE fusion protein under the control of the trp

promoter). Hybrid promoters such as the tac promoter (isolatable from plasmid pDR540 (ATCC-37282)) are also suitable. Still other bacterial promoters, whose nucleotide sequences are generally known, may be ligated to DNA encoding the protein of the instant invention, using linkers or adapters to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno sequence operably-linked to the DNA encoding the desired polyproteins. These examples are illustrative rather than limiting.

The proteins used in this invention may be synthesized either by direct expression or as a fusion protein comprising the protein of interest as a translational fusion with another protein or proteins. For example, a glutathione-S-transferase (GST)-Smad6 or Smad7 fusion protein can be synthesized essentially as described in Smith & Johnson, *Gene*, 67, 31, 1988, herein incorporated by reference. Fusion partners can be removed by enzymatic or chemical cleavage. It is often observed in the production of certain proteins in recombinant systems that expression as a fusion protein prolongs the lifespan, increases the yield of the desired protein, or provides a convenient means of purifying the protein. This is particularly relevant when expressing mammalian proteins in procaryotic hosts. A variety of peptidases (e.g. enterokinase and thrombin) which cleave a polyprotein at specific sites or digest the proteins from the amino or carboxy termini (*e.g.* diaminopeptidase) of the protein chain are known. Furthermore, particular chemicals (*e.g.* cyanogen bromide) will cleave a polyprotein chain at specific sites. The skilled artisan will appreciate the modifications necessary to the amino acid sequence (and synthetic or semi-synthetic coding sequence if recombinant means are employed) to incorporate site-specific internal cleavage sites. *See e.g.*, P. Carter, "Site Specific Proteolysis of Fusion

Proteins", Chapter 13, in Protein Purification: From Molecular Mechanisms to Large Scale Processes, American Chemical Society, Washington, D.C. (1990).

In addition to procaryotes, a variety of amphibian expression systems such as frog oocytes, and mammalian cell systems can be used in the production of recombinant Smad6 or Smad7 polypeptides. The choice of a particular host cell depends to some extent on the particular expression vector used. Exemplary mammalian host cells suitable for use in the present invention include HepG-2 (ATCC HB 8065), CV-1 (ATCC CCL 70), LC-MK_2 (ATCC CCL 7.1), 3T3 (ATCC CCL 92), CHO-K1 (ATCC CCL 61), HeLa (ATCC CCL 2), RPMI8226 (ATCC CCL 155), H4IIEC3 (ATCC CCL 1600), C127I (ATCC CCL 1616), HS-Sultan (ATCC CCL 1884), and BHK-21 (ATCC CCL 10), for example.

A wide variety of vectors are suitable for transforming mammalian host cells. For example, the pSV2-type vectors comprise segments of the simian virus 40 (SV40) genome required for transcription and polyadenylation. A large number of plasmid pSV2-type vectors have been constructed, such as pSV2-gpt, pSV2-neo, pSV2-dhfr, pSV2-hyg, and pSV2- -globin, in which the SV40 promoter drives transcription of an inserted gene. These vectors are widely available from sources such as the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852, or the Northern Regional Research Laboratory (NRRL), 1815 N. University Street, Peoria, Illinois, 61604.

Promoters suitable for expression in mammalian cells include the SV40 late promoter, promoters from eukaryotic genes, such as, for example, the estrogen-inducible chicken ovalbumin gene, the interferon genes, the glucocorticoid-inducible tyrosine aminotransferase gene, the thymidine kinase gene promoter, and the promoters of the major early and late adenovirus genes.

Plasmid pRSVcat (ATCC 37152) comprises portions of a long terminal repeat of the Rous Sarcoma virus, a virus known to infect chickens and other host cells. This long terminal repeat contains a promoter which is suitable for use in the vectors of this invention. H. German *et al.*, *Porch. Nat. Acad. SCI. (USA)*, 79, 6777 (1982). The plasmid pMSVi (NRRL B-15929) comprises the long terminal repeats of the Murine Sarcoma virus, a virus known to infect mouse and other host cells. The mouse metallothionein promoter has also been well characterized for use in eukaryotic host cells and is suitable for use in the present invention. This promoter is present in the plasmid pdBPV-MMTneo (ATCC 37224) which can serve as the starting material for the construction of other plasmids.

Transfection of mammalian cells with vectors can be performed by a plurality of well-known processes including, but not limited to, protoplast fusion, calcium phosphate co-precipitation, electroporation and the like. *See, e.g.*, Maniatis *et al.*, *supra*.

Some viruses also make appropriate vectors. Examples include the adenoviruses, the adeno-associated viruses, vaccinia viruses, herpes viruses, baculoviruses, and rous sarcoma viruses, as described in U.S. Patent 4,775,624, entirely incorporated herein by reference.

Eucaryotic microorganisms such as yeast and other fungi are also suitable host cells. The yeast *Saccharomyces cerevisiae* is the preferred eucaryotic microorganism. Other yeasts such as *Kluyveromyces lactis* and *Pichia pastoris* are also suitable. For expression in *Saccharomyces*, the plasmid YRp7 (ATCC-40053), for example, may be used. *See, e.g.*, L. Stinchcomb *et al.*, *Nature*, 282, 39 (1979); J. Kingsman *et al.*, *Gene*, 7, 181 (1979); S. Tschemper *et al.*, *Gene*, 10, 157 (1980). Plasmid YRp7 contains the TRP1 gene which provides a selectable marker for use in a trp1 auxotrophic mutant.

An expression vector carrying a cloned or isolated or endogenous Smad6 or Smad7 genomic or coding sequence or fragment thereof (e.g., SEQ ID NO: 1 or 3) is transformed or transfected into a suitable host cell using standard methods. Cells that contain the vector are propagated under conditions suitable for expression of a recombinant Smad6 or Smad7 polypeptide. For example, if the recombinant gene or fragment thereof has been placed under the control of an inducible promoter, suitable growth conditions would incorporate the appropriate inducer. The recombinantly-produced protein may be purified from cellular extracts of transformed cells by any suitable means.

In a suitable process for protein purification, a Smad6 or Smad7 gene or fragment thereof is modified at the 5' end to incorporate several histidine codons. This modification produces an "histidine tag" at the amino terminus of the encoded protein, that enables a single- or few-step protein purification method [i.e. "immobilized metal ion affinity chromatography" (IMAC)], as described in U.S. Patent 4,569,794, entirely incorporated by reference. The IMAC method enables rapid isolation of substantially pure recombinant Smad6 or Smad7 polypeptide starting from a crude extract of cells that express a modified recombinant protein, as described above.

The nucleic acids used in the present invention (e.g. SEQ ID NOS: 1 and 3) and related nucleic acid molecules may be produced by chemical synthetic methods. The synthesis of nucleic acids is well known in the art. *See, e.g.*, E.L. Brown, R. Belagaje, M.J. Ryan, and H.G. Khorana, *Methods in Enzymology*, 68:109-151 (1979). Nucleic acids, including those disclosed herein, could be generated using a conventional DNA synthesizing apparatus, such as the Applied Biosystems Model 380A or 380B DNA synthesizers (Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404) using phosphoramidite chemistry, thereafter

ligating the fragments so as to reconstitute the entire gene. Alternatively, phosphotriester chemistry may be employed to synthesize the nucleic acids of this invention. (*See, e.g.*, M.J. Gait, ed., Oligonucleotide Synthesis, A Practical Approach, (1984)).

In an alternative methodology, e.g., polymerase chain reaction (PCR), the nucleic acid sequences disclosed and described herein, can be produced from a plurality of starting materials. For example, starting with an RNA or cDNA preparation (e.g., a cDNA library) derived from a tissue that expresses a Smad6 or Smad7 gene, suitable oligonucleotide primers complementary to SEQ ID NO: 1, SEQ ID NO: 3, or to a sub-region therein, for example, are prepared as described in U.S. Patent No. 4,889,818, hereby incorporated by reference. Other suitable protocols for the PCR are disclosed in PCR Protocols: A Guide to Method and Applications, Ed. Michael A. Innis et al., Academic Press, Inc. (1990). Using PCR, any region of the Smad6 or Smad7 gene(s) can be targeted for amplification such that full or partial length gene sequences may be produced.

The ribonucleic acids used in the present invention may be prepared using polynucleotide synthetic methods discussed *supra,* or they may be prepared enzymatically, for example, using RNA polymerase to transcribe a Smad6 or Smad7 DNA template.

The most preferred systems for preparing the ribonucleic acids used in the present invention employ the RNA polymerase from the bacteriophage T7 or the bacteriophage SP6. These RNA polymerases are highly specific, requiring the insertion of bacteriophage-specific sequences at the 5' end of the template to be transcribed. *See*, Maniatis *et al.*, *supra*.

This invention also requires nucleic acids, RNA or DNA, that are complementary to Smad6 or Smad7 genes or fragments thereof, for example, SEQ ID NOS: 1 or 3.

Aspects of the present require recombinant DNA cloning vectors and expression vectors comprising the nucleic acids used in the present invention. The preferred nucleic acid vectors are those which comprise DNA, for example, SEQ ID NOS: 1 or 3 or a subregion therein.

The skilled artisan understands that choosing the most appropriate cloning vector or expression vector depends upon a number of factors including the availability of restriction enzyme sites, the type of host cell into which the vector is to be transfected or transformed, the purpose of the transfection or transformation (e.g., stable transformation as an extrachromosomal element, or integration into the host chromosome), the presence or absence of readily assayable or selectable markers (e.g., antibiotic resistance and metabolic markers of one type and another), and the number of copies of the gene desired in the host cell.

Vectors suitable to carry the nucleic acids used in the present invention comprise RNA viruses, DNA viruses, lytic bacteriophages, lysogenic bacteriophages, stable bacteriophages, plasmids, viroids, and the like. The most preferred vectors are plasmids.

When preparing an expression vector the skilled artisan understands that there are many variables to be considered, for example, whether to use a constitutive or inducible promoter. The practitioner also understands that the amount of nucleic acid or protein to be produced dictates, in part, the selection of the expression system. Regarding promoter sequences, inducible promoters are preferred because they enable high level, regulatable expression of an operably-linked gene. The skilled artisan will recognize a number of suitable promoters that respond to a variety of inducers, for example, carbon source, metal ions, and heat. Other relevant considerations regarding an expression vector include whether to include sequences for

directing the localization of a recombinant protein. For example, a sequence encoding a signal protein (e.g., SEQ ID NO: 5) preceding the coding region of a gene is useful for directing the extra-cellular export of a resulting polyprotein.

The present invention may require constructing a recombinant host cell capable of expressing the proteins used in the invention, said method comprising transforming or otherwise introducing into a host cell a recombinant DNA vector that comprises an isolated DNA sequence that encodes a protein of this invention. A suitable host cell is any eucaryotic cell that can accomodate high level expression of an exogenously introduced gene or protein, and that will incorporate said protein into its membrane structure. Vectors for expression are those which comprise a Smad6 or Smad7 gene or fragment thereof, e.g. SEQ ID NO: 1, SEQ ID NO: 3, or suitable subregion therein. Transformed host cells may be cultured under conditions well known to skilled artisans such that a protein used in the present invention is expressed, thereby producing a recombinant Smad6 or Smad7 polypeptide in the recombinant host cell.

Embodiments of a Smad6 and Smad7 DNA sequence are disclosed herein by SEQ ID NOS: 1 and 3, respectively. Those skilled in the art will recognize that owing to the degeneracy of the genetic code, numerous "silent" substitutions of nucleotide base pairs could be introduced into the sequences identified herein without altering the identity of the encoded amino acid(s) or protein or protein product.

Amino acids in a Smad6 or Smad7 polypeptide used in the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at

every residue in the molecule. The resulting mutant molecules are then tested for biological activity. Sites that are critical for ligand-protein binding can also be identified by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith et al., J. Mol. Biol. 224:899-904 (1992) and de Vos et al. Science 255:306-312 (1992)).

A Smad6 or Smad7 polypeptide can further comprise a polypeptide encoded by 4-300 contiguous amino acids of SEQ ID NO: 2 or SEQ ID NO: 4, or any range or value therein.

Also contemplated by the present invention is the use of proteins that are functionally related to Smad6 or Smad7. For example, proteins that are functionally related to SEQ ID NO: 2 may be produced by conservative amino acid substitutions, deletions, or insertions, at one or more amino acid positions within Smad6 or Smad7, in accordance with Table 1 presented herein.

Modifications of Smad6 or Smad7 polypeptides made in accordance with Table 1 are generally expected to retain the biological activity of the parent molecule based on art recognized substitutability of the amino acids specified in Table 1 (*See e.g.* M. Dayhoff, In Atlas of Protein Sequence and Structure, Vol. 5, Supp. 3, pgs 345-352, 1978). Smad6 functionality is easily tested, for example, in an assay such as that described in Example 1B herein.

ORIGINAL RESIDUE	EXEMPLARY SUBSTITUTIONS
ALA	SER, THR
ARG	LYS
ASN	HIS, SER
ASP	GLU, ASN
CYS	SER
GLN	ASN, HIS
GLU	ASP, GLU
GLY	ALA, SER
HIS	ASN, GLN
ILE	LEU, VAL, THR
LEU	ILE, VAL
LYS	ARG, GLN, GLU, THR
MET	LEU, ILE, VAL
PHE	LEU, TYR
SER	THR, ALA, ASN
THR	SER, ALA
TRP	ARG, SER
TYR	PHE
VAL	ILE, LEU, ALA
PRO	ALA

Fragments of the proteins used in the present invention may be generated by any number of suitable techniques, including chemical synthesis of any portion of Smad6 or Smad7 (e.g. SEQ ID NO: 2 or SEQ ID NO:4), proteolytic digestion of said proteins, or most preferably, by recombinant DNA mutagenesis techniques, well known to the skilled artisan. *See. e.g.* K. Struhl, "Reverse biochemistry: Methods and applications for synthesizing yeast proteins *in vitro*," *Meth. Enzymol.* 194, 520-535. For example, in a preferred method, a nested set of deletion mutations are introduced into a gene encoding Smad6 or Smad7 (e.g. SEQ ID NOS: 1 or 3), or gene fragment thereof such that varying

amounts of the protein coding region are deleted, either from the amino terminal end, or from the carboxyl end of the protein molecule. This method can also be used to create internal fragments of the intact protein in which both carboxyl and amino terminal ends are removed. Several appropriate nucleases can be used to create such deletions, for example *Bal*31, or in the case of a single stranded nucleic acid molecule, mung bean nuclease. For simplicity, it is preferred that the Smad6 or Smad7 gene be cloned into a single-stranded cloning vector, such as bacteriophage M13, or equivalent. If desired, the resulting deletion fragments can be subcloned into any suitable vector for propagation and expression in any suitable host cell.

Functional fragments of the Smad6 or Smad7 polypeptide of this invention may be produced as described above, preferably using cloning techniques to engineer smaller versions of the Smad6 or Smad7 gene, lacking sequence from the 5′ end, the 3′ end, from both ends, or from an internal site. Smaller fragments of the genes or gene fragments of this invention can be used as a template to produce the encoded proteins.

Those skilled in the art will recognize that the Smad6 or Smad7 gene used in the present invention could be obtained by a plurality of recombinant DNA techniques including, for example, hybridization, polymerase chain reaction (PCR) amplification, or *de novo* DNA synthesis.(*See e.g.*, T. Maniatis *et al.* Molecular Cloning: A Laboratory Manual, 2d Ed. Chap. 18 (1989)).

Methods for constructing cDNA libraries in a suitable vector such as a plasmid or phage for propagation in procaryotic or eucaryotic cells are well known to those skilled in the art. [*See e.g.* Maniatis *et al. Supra*]. Suitable cloning vectors are well known and are widely available.

The Smad6 or Smad7 gene, or fragments thereof, can be isolated from any tissue in which said gene is expressed. In one method for gene isolation, mRNA is isolated from a suitable tissue that expresses Smad6 or Smad7, and first strand cDNA synthesis is carried out. Second round DNA synthesis can be carried out for the production of the second strand. If desired, the double-stranded cDNA can be cloned into any suitable vector, for example, a plasmid, thereby forming a cDNA library. Oligonucleotide primers targeted to any suitable region of the sequences disclosed herein can be used for PCR amplification of Smad6 or Smad7 genes. *See e.g.* PCR Protocols: A Guide to Method and Application, Ed. M. Innis et al., Academic Press (1990). The PCR amplification comprises template DNA, suitable enzymes, primers, and buffers, and is conveniently carried out in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). A positive result is determined by detecting an appropriately-sized DNA fragment following agarose gel electrophoresis.

The present invention is also further described by reference to the following examples, which are not intended to limit the invention, but are to provide more specific, non-limiting embodiments, which support the full scope of the present invention as described herein.

EXAMPLE 1
SMAD GENE OVER-EXPRESSION

To elucidate their role on endothelial cell anticoagulant and profibrinolytic activities, the effects of Smad6 and Smad7 over-expression on two well-characterized markers of endothelial cell function, thrombomodulin (TM) and plasminogen activator inhibitor-1 (PAI-1), were examined. Both markers are known to be responsive to TGFβ, with TM being suppressed and PAI-1 being activated.

The experiments were conducted in two independent endothelial cell lines, SVHA-1 (an SV40 transformed human aortic endothelial cell line) and ECV304 (a spontaneously transformed human umbilical vein endothelial cell line) (ATCC CRL-1998) with similar results. The cells were maintained in DMEM/F-12 (3:1), a medium comprised of a 3:1 v/v mixture of Dulbecco's Modified Eagle's Medium and Ham's nutrient mixture F-12. The basal medium was supplemented with 10 nM selenium, 50 μM 2-aminoethanol, 20 mM HEPES, 50 μg/ml gentamicin, and 5% fetal bovine serum (FBS).

A. PAI-1 Promoter activity

In order to determine the level of PAI-1 secretion and promoter activity, the PAI-1 basal promoter driving the expression of the CAT indicator gene (pOCAT2336) and a TGFβ hyper-responsive PAI-1/TRE promoter construct (3TP-lux) driving the expression of the luciferase indicator gene were used. Smad6/Ha and Smad7/Ha vectors were constructed by inserting the Smad6 coding sequence into the BamHI/Xho1 sites of HA Tag expression vector pN8e23/3xFlu2 and the Smad7 coding sequence into the EcoR1/Xho1 site of pN8e23/5xFlu2, a standard expression vectors for epitope tagging. ECV304 cells were seeded in 6-well plates to 80% confluence. DNA was transfected at a concentration of 1 μg per well for pOCAT2336 and 3TP-lux and 5 μg per well for Smad6 and Samd7 with lipofectin reagent (Gibco/Life Technologies, Gaithersburg, MD). Expressed CAT protein from the PAI-1 basal promoter construct was determined using a CAT ELISA kit (Boehringer Mannheim; Indianapolis, IN). Chemiluminescence resulting from expression of the luciferase gene was determined as a measure of the effect on the PAI-1/TRE promoter. The plates were read kinetically and data expressed in terms of promoter activity relative to control (Table 2).

These experiments demonstrated a Smad6-dependent induction of the PAI-1 promoter by both the highly TGFβ-responsive transcriptional element based on the PAI-1 promoter (p3TP-Lux) as well as with the basal PAI-1 promoter (pPAI-CAT). In particular, Smad6 over-expression resulted in a higher induction with the more TGFβ-sensitive 3TP-lux plasmid.

In addition to the ability of Smad6 over-expression to activate artificial promoter constructs, the actual level of PAI-1 secreted from the cells increased almost 5-fold (Table 3).

B. Thrombomodulin activity

Determination of thrombomodulin anticoagulant activity was done in confluent cultures of SVHA-1 cells. The cultures were washed once with Hank's Basal Salt Solution to remove serum proteins and incubated with serum-free medium (DMEM/F-12 medium, 20 mM-HEPES, pH 7.5, 50 mg/ml gentamicin, 1 μg/ml human transferrin and 1 μg/ml bovine insulin) containing 400 nM recombinant human protein (made according to techniques as set forth in U.S. Patent No. 4,981,952) and 10 nM human thrombin (Sigma; St. Louis, MO). Cultures were incubated at 37°C, and at various times medium was removed and added to an equal volume of a solution of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mg/ml BSA, and 10 U/ml hirudin. The samples were incubated in the hirudin-containing buffer for 5 minutes to inhibit thrombin activity. In all experiments, samples of the protein C/thrombin solution were incubated in wells without cells to determine basal levels of thrombin-catalyzed activation of protein C.

The amount of activated protein C generated was determined by adding chromogenic substrate (S2366) (Chromogenix, Mölndal, Sweden) to a final concentration of 0.75 mM, and measuring the change in absorbance units/minute

at 405 nm in a kinetic micro-titer plate reader. Results are expressed in terms of maximal response to TGFβ (Table 4).

The amount of activated protein C generated was directly related to the level of surface TM. The over-expression of Smad6 resulted in a suppression of TM activity on the surface of the cells, to approximately 50% that observed with TGFβ.

TABLE 2

	Relative promoter Activity
plasmid 3TP-Lux	
Control	1.0 ± 0.5
+ Smad6	7.7 ± 0.2
plasmid PAI-CAT	
Control	1.0 ± 0.2
+ Smad6	2.7 ± 0.03

TABLE 3

	Relative Level of PAI-1
Control	1.0 ± 0.5
+ Smad6	4.7 ± 0.1

TABLE 4

	TM Activity (% maximal TGFβ response)
Control (untreated)	0 ± 21
TGFβ treated	100 ± 25
SMAD-6 Transfected	52 ± 21

EXAMPLE 2

EFFECT OF INHIBITING SMAD WITH ANTISENSE DNA

The oligonucleotides used in antisense experiments were synthesized using phosphorothioates and C-5 propyne pyrimidines. Antisense oligodeoxynucleotides (oligos) were designed to hybridize to the region of the Smad6 or the Smad7 mRNA encompassing the initial ATG. The antisense oligodeoxynucleotide for Smad6, was 5'-GGTTTGCCCATTCTGGACAT-3' (SEQ ID NO:5). The sequence of the sense strand control oligodeoxynucleotide for Smad6, was 5'-ATGTCCAGAATGGGCAAACC-3' (SEQ ID NO:6). The antisense oligodeoxynucleotides for Smad7, was 5'-GATCGTTTGGTCCTGAACAT-3' (SEQ ID NO:7). The sense strand control oligodeoxynucleotide for Smad7, was 5'-ATGTTCAGGACCAAACGATC-3' (SEQ ID NO:8).

Cells were plated in 96-well plates at a density of 2000 or 5000 cells/well and allowed to attach overnight in DMEM/F-12 5% FBS. After washing monolayers with serum free medium (SFM), 1 nmol of each oligo was introduced in 100 µl of SFM. Control wells containing SFM with vehicle alone were included in addition to the sense strand oligo controls. After an overnight incubation in the presence of oligos, test wells were rinsed with SFM and re-charged with oligo overnight as above. On the fourth day each experimental condition was treated with or without TGFβ at a concentration of 1 ng/ml (a concentration found to be optimal for PAI-1 and TM response). PAI-1 levels were assayed 16 hours later from the culture supernatant using a commercially available PAI-1 ELISA kit (American Diagnostica Inc., Greenwich, CT). Cell surface TM levels were assayed in SVHA-1 cells indirectly by measuring the ability of the cell surface TM to activate human protein C.

After the conditioned medium was removed for the PAI-1 assay it was replaced with 100 µl of SFM containing 25 µg/ml human protein C and 0.5 units/ml thrombin. After 1

hour incubation at room temperature, 75 µl aliquots were removed to 96-well plates, each test well containing 50 µl of 10 U/ml hirudin in activation buffer (20 mM tris pH 7.4, 150 mM NaCl), and incubated 5-10 minutes with agitation. Activated human protein C was then assayed (as a measure of TM activity) by adding 50 µl of the chromogenic substrate S2366 and measuring the change in absorbance at 405 nm on a 5 minute kinetic run.

As shown in Table 5, antisense to Smad6 blocked the TGFβ-dependent suppression of TM surface levels, as measured by the ability of the cells to support the thrombin-dependent activation of human protein C. As shown in Table 6, antisense to Smad6 suppressed the TGFβ-dependent activation of PAI-1.

TABLE 5

TM activity (% of untreated)

Treatment	control	10 µM sense	10 µM antisense
Untreated	100 ± 3	100	100
0.01 ng/ml TGFβ	91 ± 7	82	99 ± 3
0.1 ng/ml TGFβ	76 ± 2	77 ± 7	97 ± 4
1.0 ng/ml TGFβ	59 ± 4	62 ± 6	92 ± 2

TABLE 6

Sample	PAI-1 Induction (% of TGFβ-treated)
TGFβ (10 ng/ml)	100 ± 6
TGFβ plus 10 µM sense	98 ± 6.4
TGFβ plus 10 µM antisense	36 ± 6.7

EXAMPLE 3

SMAD GENE EXPRESSION IN HUMAN ENDOTHELIUM

The expression patterns for Smad6 and Smad 7 were examined in multiple samples of normal and diseased human arterial endothelium using immunohistochemical and *in situ* hybridization techniques. Table 7 reports the percentage of such samples that were positive for expression for the indicated gene.

A. Immunohistochemistry

Human tissues collected from autopsy or surgery were fixed overnight in Zn buffered formalin and then transferred to 70% ethanol prior to processing through paraffin. Five μm sections were microtomed and the slides baked overnight at 60°C. The slides were then deparaffinized in xylene and rehydrated through graded alcohols to water. Antigen retrieval was performed by immersing the slides in tissue unmasking solution (Accurate Chemical) for 10 min. at 90°C (in a water bath), cooling at room temperature for 10 min., washing in water and then proceeding with immunostaining. All subsequent staining steps were performed on an automated stainer; incubations were done at room temp., and Tris buffered saline (20mM Tris, 150mM Na(1)) plus 0.05% Tween 20, pH 7.4 was used for all washes and diluents. Thorough washing was performed after each incubation. Slides were blocked with protein blocking solution for 5 min.; after washing, 10 μg/ml of the particular Smad antibody (or irrelevant control antibody) was added to the slides and incubated for 30 minutes. A biotinylated secondary antibody plus streptavidin-horseradish peroxidase was then utilized along with a chromagenic peroxide substrate to detect the bound antibody complexes. The slides were briefly counterstained with hematoxylin, removed from the autostainer and dehydrated

through graded alcohols to xylene. The slides were coverslipped with a permanent mounting media and reviewed using light microscopy to evaluate the intensity and localization of the staining.

B. In situ hybridization

Zinc formalin fixed paraffin embedded tissues were microtomed at 5 μm and placed in an oven overnight at 60°C. The hybridization protocol followed was the Super Sensitive mRNA Detection System Kit from Biogenex. Briefly, slides were deparaffinized in xylene and rehydrated through graded alcohols to water (95%, 70% alcohols and water had RNAse block (Biogenex) added). Tissues were then digested with proteinase K solution (kit component) at room temperature for 15 minutes. After rinsing in TBS (pH 9.5) with RNAse block, the sections were dehydrated through graded alcohols and dried at room temperature. The fluorescein labeled riboprobes (both sense and anti-sense) were diluted in the kit's hybridization solution and added to the tissue and covered with a RNAse-free coverslip. Control slides had only hybridization solution (no probe) added. Hybridization was performed in a humidified thermal cycler for 10 minutes at 95°C and then 2 hours at 37°C. After several Tris washes to remove the coverslips and hybridization solution, the slides were then placed on the autostainer and the hybridized probes detected using an anti-fluorescein primary antibody, a BCIP/NBT substrate, and a nuclear fast red counterstain.

By both immunohistochemistry and *in situ* hybridization, Smad6 gene expression was observed to be significantly increased in 13 of 14 samples attained from the endothelium of atherosclerotic lesions. Smad6 was not found to be consistently expressed in normal, non-diseased vessels. In contrast, Smad7 was not consistently found

expressed in diseased vessels but was consistently expressed in normal vessels.

These data associating Smad6 with vessel disease and Smad7 with normal vessels, along with the functional data, suggest that Smad6 contributes to vascular disease, and further that suppression of Smad6 would be useful in the treatment of this disorder. The data also suggest that Smad7 protects against vascular disease.

TABLE 7

DIFFERENTIAL EXPRESSION OF SMAD6 AND SMAD7

Tissue Sample	Numbers	CD34[a]	Smad6	Smad7
Normal Artery	35	100%	10%	90%
Atherosclerotic Plaque	35	100%	70-75%	5-10%

[a] CD34 is a marker of endothelial cells

EXAMPLE 4

SMAD GENE EXPRESSION IN HUMAN MYOCYTES

Smad gene expression was also determined in myocytes from hearts of normal patients as compared to those with congestive heart failure by means of immunohistochemical and *in situ* hybridization (conducted as in Example 3). These experiments demonstrated that Smad6 is highly over-expressed in the myocytes of hearts of patients with congestive heart failure.

Because of the well described role of TGFβ in promoting myocyte hypertrophy and dysfunction, agents that suppress Smad6 and thus TGFβ secretion, including kinase inhibitors, have a positive benefit in the prevention and treatment of heart failure. The ability of Smad7 to antagonize the induction of TGFβ by Smad6 indicates that

agents that increase the function or level of Smad7 are useful in the treatment of heart failure.

EXAMPLE 5

EFFECT OF SMADS ON TGFβ PRODUCTION

A. TFGβ/CAT Transfection

In order to determine Smad effects on TGFβ promoter activity, a TGFβ3 promoter construct driving CAT expression (1 μg) was co-transfected into ECV304 cells with Smad6 (5μg)/pcineo vector (5 μg), Smad7 (5μg)/pcineo vector (5 μg), or both. Controls were co-transfected with 1 μg promoter and 10 μg pcineo vector (Promega, Madison, WI).

Transfection was initiated by plating ECV304 cells at 3 x 10^5 cells per well in a 6-well plate with DMEM/F12 media in 5% fetal bovine serum (FBS). The cells were allowed to attach overnight at 37°C.

Twenty μl lipofectin were diluted with serum-free medium to a total volume of 200 μl per transfection and placed at room temperature for 30 to 60 minutes. Eleven μg DNA was diluted with 200 μl of serum-free medium and mixed with the lipofectin solution. The resulting reagent mixture was incubated at room temperature for 15 minutes.

The medium was aspirated from the culture plates, and the cultures were washed twice with PBS. Two ml of medium were added per well, and the cells were incubated at 37°C 30 minutes prior to adding the lipofectin reagent dropwise to the cultures. The cultures were then incubated 4-6 hours at 37°, the medium was aspirated, and fresh DMEM/F12 5% FBS was added.

After culturing for 24 hours, the cells were washed twice with phosphate buffered saline (PBS), and serum free DMEM medium containing 100 μg/ml Cohn's fractionated bovine serum albumin (BSA) and 2 ng/ml TGFβ3 (R&D Systems)

was added. The cells were incubated overnight at 37°C and supernatants were collected and stored frozen. The cells were washed twice with PBS, lysed, and expressed CAT activity was measured kinetically as in Example 1. Lysates were normalized in BCA assay measuring total protein concentration.

B. Endogenous TGFβ

Endogenous levels of TGFβ1 and TGFβ3 secreted into the supernatant of the above cultures were also evaluated by ELISA.

96-well plates were coated with 0.5 μg/ml TGFβ RII receptor (R&D systems) at 100 μl per well in PBS. Plate sealer was added and the plates were stored at 4°C overnight. The plates were then washed three times with 0.1% Tween 20 in PBS, blocked with 300 μl PBS containing 5% Tween 20 and 5% sucrose for 1-3 hours at room temp.

Latent TGFβ3 was activated by adding 0.1 ml 1N HCL to 0.5 ml supernatant, and incubating 10 minutes at room temperature. The mixture was neutralized with 0.1 ml 1.2 N NaOH and 0.5 M Hepes. The blocking mixture was removed from the prepared plates, and samples were added at 200 μl per well. Standard TGFβ3, serially diluted from 2 ng/ml to 0.016 ng/ml in PBS 3% BSA, was added at 200 μl per well.

The plates were incubated for two hours and washed as before. Rabbit anti-TGFβ3 (Santa Cruz catalog# SC-082) was prepared at 1 μg/ml in PBS 3% BSA, added to the plates at 100 μl per well, incubated for one hour at room temp., and washed as before. Goat anti-Rabbit IgG alkaline phosphate conjugate at 1/250 dilution in PBS 3% goat serum was added to the plates at 100 μl per well, incubated one hour, and washed as before. One PnPP tablet (Sigma) was prepared in 3 ml H_20, added at 100 μl per well. The plates were incubated for 20-30 minutes and read at OD 405 nm.

Latent TGFβ1 was activated as described for TGFβ3 and assayed according to directions in a commercial TGFβ1 ELISA kit (R&D Systems).

Results show that in addition to the role of Smads in the signaling of TGFβ, Smad6 induces TGF promoters and the secretion of TGFβ. As shown in Table 8 (promoter) and Table 9 (secretion), Smad6 induced both the promoter activity and the secretion of TGFβ3 by around three fold. (Similar results were observed specifically for TGFβ1 as well). In contrast, Smad7 had no significant affect on TGFβ promoter activity or secretion, but could block the increase in TGFβ secretion observed with Smad6.

Additionally, the ability of Smad6 to increase endogenous TGFβ secretion and promoter activity could be blocked by the addition of a kinase inhibitor 24 hours prior to the assay. Staurosporin inhibited Smad6-induced TGFβ3 promoter activity, as measured by a decrease in CAT expression, and the PKC specific inhibitor decreased the endogenous secretion of Smad6-induced TGFβ3 measured directly (Table 10).

TABLE 8

TGFβ PROMOTER ACTIVITY IN TRANSFECTED HUMAN ENDOTHELIAL CELLS

Sample	CAT Activity (OD)
Control	0.259 ± 0.052
+Smad6	0.772 ± 0.27
+SMAD7	0.353 ± 0.12
+Smad6 +SMAD7	0.397 ± 0.05

TABLE 9

TGFβ SECRETION FROM HUMAN ENDOTHELIAL CELLS

Sample	Secreted TGFβ3 (pg/nl)
Control	77 ± 20
+Smad6	257 ± 25
+SMAD7	79 ± 7.5
+Smad6 +SMAD7	24 ± 8

TABLE 10

KINASE INHIBITION OF SMAD6-INDUCED ACTIVITY

	Protein Kinase C	Response (pg/ml TGFβ3)
Control	-	76.7 ± 20
	+	88.7 ± 15
+Smad6	-	257 ± 25
	+	66.5 ± 4.5

	Staurosporin (10 nm)	CAT Activity (OD)
Control	-	0.179 ± 0.036
	+	0.113 ± 0.047
+Smad6	-	0.83 ± 0.155
	+	0.18 ± 0.105

We claim:

1. A method for modulating at least one TGFβ regulatable activity in at least one cell, comprising contacting said at least one cell with at least one compound that modulates expression of, or the activity of the protein product of, a nucleic acid molecule having at least 90% identity to at least 40 contiguous nucleotides of a nucleic acid selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, a nucleic acid molecule that is complementary to SEQ ID NO:1, a nucleic acid molecule that is complementary to SEQ ID NO:3, and at least one fragment thereof.

2. The method of claim 1 wherein the nucleic acid molecule has at least 95% identity to at least 40 contiguous nucleotides of a nucleic acid selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, a nucleic acid molecule that is complementary to SEQ ID NO:1, a nucleic acid molecule that is complementary to SEQ ID NO:3, and at least one fragment thereof.

3. The method of claims 1 and 2 wherein said TGFβ regulatable activity is at least one selected from the group consisting of an induction of plasminogen activator inhibitor-1 expression, an increase in plasminogen activator inhibitor-1 secretion, a suppression of thrombomodulin activity, an increase in TGFβ secretion, and a decrease in TGFβ secretion.

4. A method for modulating a TGFβ regulatable activity, comprising administering to a cell, cells, or a patient in need of such treatment, a protein encoded by a nucleic acid molecule having at least 90% identity to at least 40 contiugous nucleotides of a nucleic acid selected from the

group consisting of SEQ ID NO:1, SEQ ID NO:3, and at least one fragment thereof.

5. The method of claim 4 wherein the protein to be administered is encoded by a nucleic acid molecule having at least 95% identity to a member selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and at least one fragment thereof.

6. The method of claims 4 and 5 wherein the protein to be administered is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and at least one fragment thereof.

7. The method of claims 4-6 wherein said TGFβ regulatable activity is at least one selected from the group consisting of an induction of plasminogen activator inhibitor-1 expression, an increase in plasminogen activator inhibitor-1 secretion, a suppression of thrombomodulin activity, an increase in TGFβ secretion, and a decrease in TGFβ secretion.

8. A method for modulating a TGFβ regulatable activity, comprising administering to at least one cell, an organism, or a patient in need of such treatment, an antisense nucleic acid molecule having a nucleotide sequence complementary to at least 10 contiguous nucleotides of an mRNA transcribed from a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and at least one fragment thereof, wherein said antisense nucleic acid molecule hybridizes to said contiguous sequence such that translation of said mRNA is inhibited.

9. The method of claim 8 wherein the antisense nucleic acid molecule is selected from the group consisting of at least 10 contiguous nucleotides of SEQ ID NO:5 and SEQ ID NO:7.

10. The method of claims 8 and 9 wherein said TGFβ regulatable activity is at least one selected from the group consisting of an induction of plasminogen activator inhibitor-1 expression, an increase in plasminogen activator inhibitor-1 secretion, a suppression of thrombomodulin activity, an increase in TGFβ secretion, and a decrease in TGFβ secretion.

11. A method for the prevention of a disease arising from cellular effects induced by TGFβ, said method comprising administering to a patient in need of such treatment a compound that modulates expression of, or the activity of the protein product of, a nucleic acid molecule comprising at least 40 nucleotides selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and fragments thereof.

12. A method for treating a disease in which TGFβ is responsible for inducing cellular effects that lead to said disease, said method comprising administering to a patient in need of such treatment, a compound that modulates expression of, or the activity of the protein product of, a nucleic acid molecule comprising at least 40 contiguous nucleotides selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and at least one fragment thereof.

13. A method according to claim 11 wherein the disease to be prevented is at least one selected from the group consisting of myocardial infarction, congestive heart failure, dilated cardiomyopathy, deep venous thrombosis, disseminated intravascular thrombosis, stroke, sepsis, injuries involving major tissue damage and trauma, systemic inflammatory response syndrome, sepsis syndrome, septic shock, multiple organ dysfunction syndrome (including DIC), atherosclerotic plaque rupture, and associated sequela arising therefrom.

14. A method according to claim 12 wherein the disease to be treated is at least one selected from the group consisting of myocardial infarction, congestive heart failure, dilated cardiomyopathy, deep venous thrombosis, disseminated intravascular thrombosis, sepsis, injuries involving major tissue damage and trauma, systemic inflammatory response syndrome, sepsis syndrome, septic shock, multiple organ dysfunction syndrome (including DIC), atherosclerotic plaque rupture, and associated sequela arising therefrom.

15. A method of modulating TGFβ secretion comprising administering to at least one cell, an organism, or a patient in need of such treatment, at least one compound that increases expression of, or the activity of the protein product of, a nucleic acid molecule comprising at least 40 contiguous nucleotides selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and at least one fragment thereof.

16. A method of inhibiting TGFβ secretion, comprising administering to at least one cell, an organism, or a patient in need of such treatment, a kinase inhibitor.

17. The method of claim 16 wherein the kinase inhibitor is a protein kinase C inhibitor.

18. A method for identifying a compound that induces thrombomodulin activity or blocks suppression of thrombomodulin activity on the surface of cells comprising: contacting a sample of cells with a test compound; measuring the amount of activated protein C generated by the sample of cells; comparing said amount to the amount of activated protein C generated in a sample of cells not exposed to test compound but otherwise identically treated such that if the level of thrombomodulin activity determined in the treated

sample is higher than the untreated sample, a compound which induces or blocks suppression of thrombomodulin activity has been identified.

19. The method of claim 18 wherein said sample of cells has been transfected with a vector allowing for the expression of a protein encoded by a nucleic acid molecule having at least 70% identity to a member selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and fragments thereof.

20. The method of claims 18 and 19 wherein said sample of cells is treated with TGFβ.

21. The use of a compound that modulates expression of, or the activity of the protein product of, a nucleic acid molecule molecule selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and fragments thereof for the manufacture of a medicament for the prevention of a disease in which TGFβ is responsible for inducing cellular effects that lead to said disease.

22. The use of a compound that modulates expression of, or the activity of the protein product of, a nucleic acid molecule molecule selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and fragments thereof, for the manufacture of a medicament for the treatment of a disease in which TGFβ is responsible for inducing cellular effects that lead to said disease.

23. The use of a compound having at least 90% identity to a member selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and fragments thereof, for the manufacture of a medicament for the prevention of a disease in which TGFβ is

responsible for inducing cellular effects that lead to said disease.

24. The use of a compound having at least 90% identity to a member selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and fragments thereof, for the manufacture of a medicament for the treatment of a disease in which TGFβ is responsible for inducing cellular effects that lead to said disease.

25. The use of a compound which inhibits protein kinase C for the manufacture of a medicament for the treatment or prevention of a disease in which TGFβ is responsible for inducing cellular effects that lead to said disease.

26. The use of a compound as claimed in Claim 21, 23, or 25 for the manufacture of a medicament for the prevention of disease wherein the disease to be prevented is selected from the group consisting of myocardial infarction, congestive heart failure, dilated cardiomyopathy, deep venous thrombosis, disseminated intravascular thrombosis, stroke, sepsis, injuries involving major tissue damage and trauma, systemic inflammatory response syndrome, sepsis syndrome, septic shock, multiple organ dysfunction syndrome (including DIC), atherosclerotic plaque rupture, and associated sequela arising therefrom.

27. The use of a compound as claimed in claims 22, 24, or 25 for the manufacture of a medicament for the treatment of disease wherein the disease to be treated is selected from the group consisting of myocardial infarction, congestive heart failure, dilated cardiomyopathy, deep venous thrombosis, disseminated intravascular thrombosis, stroke, sepsis, injuries involving major tissue damage and trauma, systemic inflammatory response syndrome, sepsis syndrome,

septic shock, multiple organ dysfunction syndrome (including DIC), atherosclerotic plaque rupture, and associated sequela arising therefrom.

28. A method for modulating at least one TGFβ regulatable activity in at least one cell or an organism comprising contacting said at least one cell or organism with at least one compound that modulates expression of, or the activity of the protein product of, a nucleic acid molecule having at least 90% identity to at least 40 contiguous nucleotides of a nucleic acid selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, a nucleic acid molecule that is complementary to SEQ ID NO:1, a nucleic acid molecule that is complementary to SEQ ID NO:3, or at least one fragment thereof.

29. The method of claim 28 wherein the nucleic acid molecule has at least 95% identity to a member selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, a nucleic acid molecule that is complementary to SEQ ID NO:1, a nucleic acid molecule that is complementary to SEQ ID NO:3, and fragments thereof.

30. The method of claims 28 and 29 wherein said TGFβ regulatable activity is at least one selected from the group consisting of an induction of plasminogen activator inhibitor-1 expression, an increase in plasminogen activator inhibitor-1 secretion, a suppression of thrombomodulin activity, an increase in TGFβ secretion, and a decrease in TGFβ secretion.

31. A method for modulating a TGFβ regulatable activity, comprising administering to at least one cell or an organism, a protein encoded by a nucleic acid molecule having at least 90% identity to at least 40 contiguous

nucleotides of a nucleic acid selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and at least one fragment thereof.

32. The method of claim 31 wherein the protein to be administered is encoded by a nucleic acid molecule having at least 95% identity to at least 40 contiguous nucleotides of a nucleic acid selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and fragments thereof.

33. The method of claims 31 or 32 wherein the protein to be administered is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and at least one fragment thereof.

34. The method of claims 31-33 wherein said TGFβ regulatable activity is at least one selected from the group consisting of an induction of plasminogen activator inhibitor-1 expression, an increase in plasminogen activator inhibitor-1 secretion, a suppression of thrombomodulin activity, an increase in TGFβ secretion, and a decrease in TGFβ secretion.

35. A method for modulating a TGFβ regulatable activity, comprising administering to at least one cell, organism, or patient in need of such treatment, an antisense nucleic acid molecule having a nucleotide sequence of at least one complementary to a contiguous sequence of mRNA transcribed from a gene selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and fragments thereof wherein said antisense nucleic acid molecule hybridizes to said contiguous sequence such that translation of said mRNA is inhibited.

36. The method of claim 35 wherein the antisense nucleic acid molecule is at least 10 contiguous nucleotides selected from the group consisting of SEQ ID NO:5 and SEQ ID NO:7.

37. The method of claims 35 and 36 wherein said TGFβ regulatable activity is at least one selected from the group consisting of an induction of plasminogen activator inhibitor-1 expression, an increase in plasminogen activator inhibitor-1 secretion, a suppression of thrombomodulin activity, an increase in TGFβ secretion, and a decrease in TGFβ secretion.

38. A method of inhibiting TGFβ secretion, comprising administering to at least one cell or an organism, a kinase inhibitor.

39. The method of claim 38 wherein the kinase inhibitor is a protein kinase C inhibitor.

40. A method for identifying a compound that induces thrombomodulin activity or blocks suppression of thrombomodulin activity on the surface of cells comprising: contacting a sample of cells with a test compound; measuring the amount of activated protein C generated by the sample of cells; comparing said amount to the amount of activated protein C generated in a sample of cells not exposed to test compound but otherwise identically treated such that if the level of thrombomodulin activity determined in the treated sample is higher than the untreated sample, a compound which induces or blocks suppression of thrombomodulin activity has been identified.

41. The method of claim 40 wherein said sample of cells has been transfected with a vector allowing for the expression of a protein encoded by a nucleic acid molecule having at

least 70% identity to a member selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and fragments thereof.

42. The method of claims 40 and 41 wherein said sample of cells is treated with TGFβ.

43. Any invention described, referred to, exemplified, or shown herein.

SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06595

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07K 14/495, 14/81; A61K 38/18; C12Q 1/00
US CL :530/350; 435/7.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 435/7.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE
search terms: smad6, smad7, pai-1, TGF-beta

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LUND et al. Transforming growth factor-β is a strong and fast acting positive regulator of the level of type-1 plasminogen activator inhibitor mRNA in WI-38 human lung fibroblasts. EMBO J. 1987, Vol. 6, No. 5, pages 1281-1286, see especially page 1281, column 2, full paragraph 2; Figures 2 and 4.	1-3
X, P	AFRAKHTE et al. Induction of inhibitory smad6 and smad7 mRNA by TGF-β family members. Biochemical and Biophysical Research Communications. August 1998, Vol. 249, pages 505-511, see especially Figure 4.	1-3

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
30 JUNE 1999

Date of mailing of the international search report
04 AUG 1999

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06595

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

With respect to unity of invention PCT Rule 13.1 states:

The international application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention").

Additionally, PCT Rule 13.2 states:

Where a group of inventions is claimed in one and the same international application, the requirement of unity of invention referred to in Rule 13.1 shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

With regard to the application of PCT Rule 13, 37 CFR § 1.475 concerning unity of invention states:

(a) An international and a national stage application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention"). Where a group of inventions is claimed in an application, the requirement of unity of invention shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

(b) An international or a national stage application containing claims to different categories of invention will be considered to have unity of invention if the claims are drawn only to one of the following combinations of categories:

(1) A product and a process specially adapted for the manufacture of said product; or

(2) A product and a process of use of said product; or

(3) A product, a process specially adapted for the manufacture of the said product, and a use of the said product; or

(4) A process and an apparatus or means specifically designed for carrying out the said process; or

(5) A product, a process specially adapted for the manufacture of the said product, and an apparatus or means specifically designed for carrying out the said process.

(c) If an application contains claims to more or less than one of the combinations of categories of invention set forth in paragraph (b) of this section, unity of invention might not be present.

(d) If multiple products, processes of manufacture or uses are claimed, the first invention of the category first mentioned in the claims of the application and the first recited invention of each of the other categories related thereto will be considered as the main invention in the claims, see PCT Article 17(3)(a) and § 1.476(c).

(e) The determination whether a group of inventions is so linked as to form a single general inventive concept shall be made without regard to whether the inventions are claimed in separate claims or as alternatives within a single claim.

The inventions listed as Groups I-CDXCVIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Claims 1 and 2 recite a plurality of methods comprising contacting a cell with a compound that modulates the expression

of, or the activity of the protein product of, a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3. Smad6 (SEQ ID NO:2) is the protein product of SEQ ID NO:1. Smad7 (SEQ ID NO:4) is the protein product of SEQ ID NO:3. In order for the inventions of claim 1 to have unity of invention it is necessary that the single inventive concept be a contribution over the prior art. The inhibitory SMADs, Smad6 and Smad7, do not fulfill the requirements of unity of invention because each is known in the prior art. Furthermore, it is known in the prior art that transcription of inhibitory SMAD mRNA is induced by stimulation by TGF-β as well as by other stimuli. See, for example, HELDIN et al. (Nature, 4 December 1997, Vol. 390, pages 465-471, especially page 469, column 1, full paragraph 1). Therefore, contacting a cell with a compound that modulates the expression of a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3 does not fulfill the requirements of unity of invention because such a method is known in the prior art.

In addition, (A) Smad6 and (B) Smad7, and the nucleic acid molecules encoding them, are structurally and functionally distinct compounds and do not share the same inventive concept.

In addition, (1) modulating the expression of a nucleic acid molecule, (2) modulating the expression of the complement of said nucleic acid molecule, (3) and modulating the activity of the protein product of said nucleic acid molecule, and (4) modulating the activity of the protein product of said complement of said nucleic acid molecule do not share the same inventive concept because each is directed to functionally distinct methods and because a nucleic acid molecule and its complement encode completely unrelated proteins.

Claim 3 shares the same inventive concept with claims 1 and 2 with regard to the method but it does not share the same inventive concept with regards to the TGF-β regulatable activity. Thus there are 4 inventive concepts as follows: (i) induction of PAI-1 expression and secretion, (ii) suppression of TM activity, (iii) increased TGF-β secretion, and (iv) decreased TGF-β secretion.

It follows from the preceding analysis that claims 1-3 constitute 32 separate groups. For each of (A) and (B), Smad6 and Smad7, respectively, there are four distinct methods (1-4) directed thereto, with four distinct outcomes (i-iv).

Groups I-XXXII are represented by combinations of (A) or (B) with (1-4) and (i-iv).

Group I is considered to be the main invention and is directed to a method of inducing PAI-1 expression and secretion comprising contacting a cell with a compound that modulates the expression of a nucleic acid molecule comprising a nucleotide sequence having at least 90% identity to at least 40 contiguous nucleotides of SEQ ID NO:1.

Claims 4-7 appear to share the same inventive concept with groups I-XXXII with respect to (A) and (B), and (i-iv) but administering a protein, (A) or (B) does not share the same inventive concept with (1-4) because it is directed to a distinct method using a distinct compound.

In addition, (5) administering to cells *in vitro* does not share the same inventive concept with (6) administering to a patient *in vivo* because *in vitro* cell culture techniques are distinct from *in vivo* therapeutic methods.

It follows from the preceding analysis that claims 4-7 constitute 16 separate groups. For each of (A) and (B), Smad6 and Smad7, respectively, there are two distinct methods (5-6) directed thereto, with four distinct outcomes (i-iv).

Groups XXXIII-XLVIII are represented by combinations of (A) or (B) with (5-6) and (i-iv).

Claims 8-10 appear to share the same inventive concept with groups I-XLVIII with respect to (A) and (B), (5-6), (i-iv) but inhibiting the expression of a nucleic acid molecule with antisense nucleic acid molecules does not share the same inventive concept with inhibiting the expression of nucleic acid molecule by inhibiting any or all things distal or proximal to gene transcription.

It follows from the preceding analysis that claims 8-10 constitute 16 separate groups. For each of (A) and (B), Smad6 and Smad7, respectively, there are two distinct methods (5-6) directed thereto, with four distinct outcomes (i-iv).

Groups XLIX-LXIV are represented by combinations of (A) or (B) with (5-6) and (i-iv).

Claims 11-14 appear to share the same inventive concept with groups I-LXIV with respect to (A-B) and (1-2) but prevention or treatment of a disease does not share the same inventive concept with modulation of a TGF-β regulatable activity because there is no necessary linkage between the disease and the TGF-β regulatable activity.

In addition, (7) preventing a disease and (8) treating a disease do not share the same inventive concept because prevention is directed to avoiding the occurrence of a disease whereas treatment implies the treatment effecting an extant disease. Similarly, the diseases recited in claims 13 and 14, each of which is herein assigned a number from 9-22, would appear to have separate etiologies and therefore lack the same inventive concept.

It follows from the preceding analysis that claims 11-14 constitute 112 separate groups. For each of (A) and (B), Smad6 and Smad7, respectively, there are two distinct methods (1, 3) directed thereto, with two distinct outcomes (7-8), and fourteen (9-22) distinct disease states.

Groups LXIV-CLXXVI are represented by combinations of (A) or (B) with (1, 3), (7,8) and (9-22).

Groups CLXXVII-CLXXX (claim 15) are represented by a method of modulating TGF-β secretion in a cell *in vitro* by combinations of (A) or (B) and (1 or 3).

Groups CLXXXI-CLXXXIV (claim 15) are represented by a method of modulating TGF-β secretion in a patient *in vivo* by combinations of (A) or (B) and (1 or 3).

Groups CLXXXV-CLXXXVI (claims 16 and 17) are represented by combinations of (5) *in vitro* and (6) *in vivo* methods of inhibiting TGF-β secretion by administering a protein kinase C inhibitor. Administering a protein kinase C inhibitor does not share the same inventive concept with administering all structurally and functionally distinct compounds of indeterminate constitution that modulate a TGF-β response.

Groups CLXXXVII-CLXXXVIII (claims 18 and 20) are represented by combinations of a method of identifying a compound that either (23) induces TM activity or (24) blocks suppression of TM activity. Inducing TM activity and blocking suppression of TM activity do not share the same inventive concept because they are functionally distinct methods and one can be practiced without the other.

Groups CLXXXIX-CXC (claims 19 and 20) appear to share the same inventive concept with groups CLXXXVII-CLXXXVIII but a cell transformed with a (A) or (B), Smad6 and Smad7, respectively, expression vector does not share the same inventive concept an untransformed cell because the cells in each case a structurally distinct.

Groups CXCI- CXCIV are represented by combinations of (23, induces TM activity) or (24, blocks suppression of TM activity) with (A) or (B), Smad6 and Smad7, respectively,.

Groups CXCV-CCL (claims 21 and 26) are represented by combinations of (A) or (B), with (1) or (3), and the prevention of a disease, wherein the disease is selected from the group consisting of (9-22), see above.

Groups CCLI-CCCVI (claims 22 and 27) are represented by combinations of (A) or (B), with (1) or (3), and the treatment of a disease, wherein the disease is selected from the group consisting of (9-22), see above.

Groups CCCVII-CCCXXXIV (claims 23 and 26) are represented by combinations of (A) or (B), with the prevention of a disease, wherein the disease is selected from the group consisting of (9-22), see above.

Groups CCCXXXV-CCCLXII (claims 24 and 27) are represented by combinations of (A) or (B), with the treatment of a disease, wherein the disease is selected from the group consisting of (9-22), see above.

Groups CCCLXIII-CCCLXXVI (claims 25 and 26) are represented by combinations of the use of protein kinase C, with the prevention of a disease, wherein the disease is selected from the group consisting of (9-22), see above.

Groups CCCLXXVII-CCCXC (claims 25 and 27) are represented by combinations of the use of protein kinase C, with the treatment of a disease, wherein the disease is selected from the group consisting of (9-22), see above.

Claims 28-30 constitute 64 separate groups. For each of (A) and (B), Smad6 and Smad7, respectively, there are four distinct methods (1-4) directed thereto, with four distinct outcomes (i-iv), comprising administering to a cell *in vitro* or to an organism *in vivo*, see above.

Groups CCCXCI-CDLIV (claims 28-30) are represented by combinations of (A) or (B) with (1-4) and (i-iv), *in vitro* or *in vivo*.

Claims 31-34 constitute 16 separate groups. For each of (A) and (B), Smad6 and Smad7, respectively, there are four distinct outcomes (i-iv), comprising administering to a cell *in vitro* or to an organism *in vivo*, see above.

Groups CDLV-CDLXX (claims 31-34) are represented by combinations of (A) or (B) with (i-iv), *in vitro* or *in vivo*.

Claims 35-37 constitute 16 separate groups. For each of the antisense molecules that are antisense to nucleic acid molecules encoding either (A) or (B), Smad6 and Smad7, respectively, there are four distinct outcomes (i-iv), comprising administering to a cell *in vitro* or to an organism *in vivo*, see above.

Groups CDLXXI-CDLXXXVI (claims 35-37) are represented by combinations of (A) or (B) with (i-iv), *in vitro* or *in vivo*.

Group CDLXXXVII (claims 38 and 39) is represented by administering a protein kinase C inhibitor *in vitro*.

Groups CDLXXXVII (claims 38 and 39) is represented by administering a protein kinase C inhibitor *in vivo*.

Groups CDLXXXIX-CDXC (claims 40 and 42) are represented by combinations of a method of identifying a compound that either (23) induces TM activity or (24) blocks suppression of TM activity. Inducing TM activity and blocking suppression of TM activity do not share the same inventive concept because they are functionally distinct methods and one can be practiced without the other.

Groups CDXCI-CDXCII (claims 41 and 42) appear to share the same inventive concept with groups CDLXXXIX-CDXC but a cell transformed with a (A) or (B), Smad6 and Smad7, respectively, expression vector does not share the same inventive concept with an untransformed cell because the cells in each case a structurally distinct.

Groups CDXCIII-CDXCVII are represented by combinations of (23, induces TM activity) or (24, blocks suppression of TM activity) with (A) or (B), Smad6 and Smad7, respectively.

Group CDXCVIII does not share an inventive concept with the other groups to the extent that it is drawn to any single invention other than the above groups.